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COMPLETE SPECIFICATION

**Method and Apparatus for the Aseptic Propagation of
Microbial Cultures for Industrial Use**

We, COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANIZATION, a body corporate established under the Science and Industry Research Act, 1949, carrying on scientific and industrial research, of 314 Albert Street, East Melbourne, in the State of Victoria, Commonwealth of Australia do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:

The invention relates to a method and apparatus for the aseptic propagation of microbial cultures for industrial use.

Bacterial, yeast and mould cultures (hereinafter referred to as "microbial cultures") are used in many industrial fermentation processes, e.g. wine and beer making, and in the dairy products industry in the manufacture of cheese, yoghurt, cultured cream and cultured buttermilk. In all these processes a substrate, usually a carbohydrate, is converted by the action of the micro-organisms to acids or alcohol, or a mixture of these, or other end products, and the cultures are selected on their ability to produce the desired result.

Since various species and types of micro-organisms differ from one another qualitatively or quantitatively in their biochemical activities, it is highly desirable to maintain the selected cultures in a pure state, that is to say, free from contamination by other micro-organisms and undesirable extraneous organic or inorganic matter. For this reason, the cultures are propagated in sterile media and sterile utensils or apparatus.

In many cases the cultures are made up first in a small volume, sometimes called the

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"mother culture", from which a larger volume, sometimes called the "bulk culture", is made for the purpose of inoculating large batches of the material or substrate to be converted on an industrial scale.

In the manufacture of cheese, several species of bacteria from among the lactic streptococci and lactobacilli are used for the purpose of conversion of lactose into lactic acid as well as several other reactions all of which influence the quality of the cheese.

Cultures of these bacteria are usually called cheese starter cultures or cheese starters. Since they are required for the inoculation of every batch of milk to be converted into cheese, they have to be propagated in the cheese factories from day to day. It is essential in the propagation of these starter cultures to use aseptic techniques in order to minimise or to exclude their contact with unsterile material, including air, and thus prevent their contamination with other bacteria, yeasts, moulds and also with viruses known as bacteriophages, which are capable of lysing or virtually destroying these cultures with consequent failure of one of the main biochemical processes in the manufacture.

In industrial practice, the cheese starter cultures are usually propagated in two stages. The first, or mother culture, stage, is made up first and serves as an inoculum for the bulk culture.

The mother cultures are normally prepared in sterilized milk in sterilized flasks or other utensils. After incubation at the required temperature, the mother culture is transferred to the bulk starter vessel where it serves as an inoculum. The transfer from the mother culture to the bulk starter vessel should be done aseptically, since in spite of aseptic propagation up to this stage, con-

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amination may occur at this point, leading to a contaminated bulk culture. Under industrial conditions, a completely aseptic transfer is difficult to achieve even in the hands of trained personnel. With relatively untrained personnel, the risk of contamination is much greater. Further, it has been found necessary in cheesemaking to use at one, but several cultures in order to minimize the risk of contamination particularly with bacteriophage at all stages of culture propagation, and also in the cheese vat. A scheme has therefore been evolved, in which a so called bacteriophage-unrelated culture, or a pair of such cultures, is used on the first day, followed by another unrelated culture, or pair of such cultures, on the next day, followed by yet another unrelated culture, or pair of such cultures, on the third day. On the fourth day, the first culture, or pair of such cultures, is used again and the scheme is repeated again and again. In intensive manufacture it may be necessary to extend this rotation by using as many unrelated cultures as possible. In this way critical concentrations of bacteriophage, capable of destroying any of these cultures, can be avoided.

It follows from this scheme that not one, but many, starter cultures must be propagated in the factory and because mother cultures, unless deep-frozen, do not readily survive even in a refrigerator for more than about three days, they must be re-propagated even if not actually required for the inoculation of bulk cultures. Under prevailing conditions the risk of contamination at re-propagation is similar to that at the point of transfer from the mother culture vessel to the bulk starter vessel.

It is accordingly the principal object of this invention to substantially eliminate or reduce the risk of contamination in the propagation of mother cultures and in their transfer from the mother culture vessel to the bulk culture vessel.

A further object is the provision of apparatus by means of which a microbial culture can be transferred from one container to another while minimizing or avoiding contact with any unsterile material, including air.

Other objects and advantages of the invention will become apparent to those skilled in the art as the ensuing description proceeds.

According to one aspect of this invention, there is provided a process for the aseptic propagation of microbial cultures comprising the steps of aseptically transferring sterilized medium to a sterilized container, inoculating the medium in the container under aseptic conditions, incubating the inoculated culture under aseptic conditions, transferring a portion of the incubated culture to another

vessel under aseptic conditions, retaining portion of the incubated culture in the container to serve as an inoculum for a fresh culture, supplying fresh sterilized medium under aseptic conditions to the container, and incubating under aseptic conditions the fresh culture thus formed.

The invention also includes apparatus for the aseptic propagation of microbial cultures comprising sterilizing means, a container suitable for the growth of microbial cultures, connections between the container and a vessel into which a culture grown in the container can be passed, connections between the sterilizing means and the container, and valve means in said connections, the arrangement being such that both aseptic propagation of microbial cultures within the container and the aseptic transfer of microbial cultures from the container to the vessel can be effected, the connections between the container and the vessel being arranged so as to ensure that a portion of the contents of the container remains in the container when a culture is transferred from the container to the vessel. The apparatus preferably includes an air or gas sterilizing filter for admission of sterile air or gas to the container. Preferably, the container connections are such that the container may be readily detached from the system and re-connected thereto when desired, in such manner that the connections can be readily re-sterilized.

In order that the invention, and a practical application thereof, may be more fully understood, reference will now be made by way of example to the accompanying drawings, wherein:

Figure 1 is a side view of apparatus according to the invention for the aseptic propagation of bacterial cultures suitable for use in cheesemaking, and

Figure 2 is an enlarged view of part of the apparatus shown in Figure 1.

As shown in the drawings, there is provided an autoclave A in which steam can be generated to the required pressure and then supplied to a jacketed container M used for the propagation of mother cultures. A bulk culture vessel V (commonly known as a bulk starter vessel) is arranged to receive cultures from the container M through a two-way valve 4, a sealed joint 8 and a four-way valve 5. Connection between the autoclave A and the container M may be effected through four-way valve 2, sealed joint 10, water-jacketed manifold 16, two-way valve 4 and pressure tube 13. A steam outlet passage 19 connected between the autoclave A and valve 2 includes the four-way valve 1 which is, itself, in communication with the connection between the joint 9 and the valve 5. The latter connection is connected to a joint 11 leading

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to a three-way filter valve 6 and an air or gas filter 17. Also connected to the valve 5 is a pressure relief valve 18 and bleed. The valves 1, 3 and 6 lead in one of their positions to the exterior of the system thus providing air, steam or liquid bleeds for the system. The container M is provided with a pierceable membrane which is protected by a removable threaded sealing cap 12.

All the parts of the assembly described above are permanently attached to the autoclave A with the exception of (a) the filter valve 6 and the air or gas filter 17 which sub-assembly can be detached from, or attached to, the system via the joint 11, and (b) the water-jacketed manifold sub-assembly which includes valves 3 and 4 and the container M, the manifold being detachable from, or attachable to, the remainder of the system through the joints 8, 9 and 10. The water-jacket of the manifold is connected through joints 14 and 15 to a cooling water inlet and a cooling water outlet respectively, the inlet including a control valve 7.

In a typical process according to the invention, steam is generated in the autoclave A and this steam is allowed to pass from the autoclave through valve 1, joint 10, manifold 16, valve 4, pressure tube 13, container M, valve 3, joint 9, filter valve 6 and thence to atmosphere through the bleed. The steam may simultaneously or subsequently also be permitted to pass from the manifold 16 through valve 5 to atmosphere.

After the air has been evacuated from the system by the passage of steam through the apparatus in this manner, the air and steam outlets are closed and steam pressure to at least 15 p.s.i. gauge is developed by heating the autoclave. In this case, the steam will pass out of the system through the bleed of the relief valve 18 instead of through the bleed from the filter valve 6 as in the previous step. The valve 5 may be initially closed but later opened so as to allow the passage of steam therethrough. In this way, the valves 1, 3, 4 and 5, the various inter-connecting passages and the container M are sterilized.

By suitably adjusting the valve 1, the autoclave A is isolated from the rest of the system, allowed to cool, the lid is opened and a suitable quantity of liquid medium, for example milk, placed therein. The autoclave is then closed and heated in order to sterilize the medium therein, the air from within the autoclave being forced out by steam generated from the liquid medium. In order to thoroughly sterilize the medium in the autoclave, the steam pressure in the autoclave is allowed to rise to at least 15 p.s.i. gauge. At this stage, cooling water

is caused to circulate around the manifold 16, entering the same through joint 14 and leaving the same through joint 15.

After the liquid medium in the autoclave has thus been thoroughly sterilized, it is allowed to cool to a temperature of about 95°F and transferred through valve 2, manifold 16 (which has meanwhile been cooled to about 65°F) and valve 4 to the container M, valves 1 and 3 being adjusted to allow a return passage for vapour between the container M and the autoclave.

Since air, oxygen, carbon dioxide or other gas may be required for active growth of micro-organisms in the container M, this vessel is then isolated from the autoclave and is connected through valves 3 and 6 with the air or gas filter 17. This filter is preferably constructed to have, as a filtering medium, a compacted layer of cotton wool or any other material which will permit the passage of air or gas, but will trap and thus remove any micro-organisms or bacteriophages. The filter itself, together with its isolating valve 6, is removable for sterilization in a sterilizing oven before being fitted to the assembly. Air or gas, after passing through the filter, will not contain any viable matter and can therefore be considered as sterile for the purpose of this invention.

After the sterile air or gas has thus been admitted to the container M through the filter, the container is isolated from the rest of the system and from the air or gas supply by closing the valves 3 and 4, and the manifold is flushed out with cold water which passes into the autoclave through the valve 2 and then passes through valve 1, joint 10, joint 8 and finally leaves the system through the valve 5. The container and its associated manifold is then detached from the rest of the system through joints 8, 9, 10, 14 and 15 for aseptic inoculation and incubation.

As will appear hereinafter, it is necessary to carry out a special inoculation procedure once only, as thereafter, medium transferred to the container will be automatically inoculated by a portion of the incubated culture remaining in the container from a previous run.

For the purpose of the initial inoculation, the sterile liquid medium in the container is brought to the required temperature and is inoculated with a pure culture of the selected micro-organism by means of a hypodermic syringe, the needle of which is introduced into the container through the pierceable membrane which is exposed after removal of the cap 12. The contents of the container are then incubated at a suitable temperature until sufficient microbial population develops to serve as a mother culture.

- In this way, a number of mother cultures of the same micro-organism or of different strains or different species of micro-organisms can be prepared. A mother culture prepared in this way can either be used immediately for inoculating the bulk starter or can be deep-frozen and stored in that state until required.
- When a particular mother culture is required for use as an inoculant for the production of a bulk starter culture, the container containing the mother culture concerned, whether in the liquid or the deep-frozen state, is re-connected together with its manifold to the system through the joints 8, 9, 10, 14 and 15. The residual flushing water is then drained from the autoclave through valve 2.
- After the container has been reconnected to the system, the valves 3 and 4 remain closed and steam generated in the autoclave is caused to pass through valve 1, manifold 16, joint 8, valve 5 and thence to atmosphere. This flow of steam evacuates air from the autoclave and the connecting passages and valves. The autoclave is then further heated to raise the steam pressure to at least 15 p.s.i. gauge in order to thoroughly sterilize all the connections up to the container valves 3 and 4. For this purpose, the valve 3 is adjusted to allow steam to bleed to atmosphere therethrough and the valve 5 is adjusted to allow steam from the manifold to pass through the valve to the bleed of the relief valve 18. Such a sterilizing procedure should be maintained for a period of at least 15 minutes.
- Whilst this sterilization is being effected, the container M is held at a suitable temperature. If the mother culture in the container was in a frozen state when the container was connected to the system, the jacket of the container is warmed up to a suitable temperature, e.g. 95°F, in order to thaw the contents completely.
- On completion of the sterilization, the valves 1 and 3 are adjusted so as to allow steam generated under pressure in the autoclave to pass through the valve 1, joint 9 and valve 3 into the interior of the container M. At the same time, valve 4 is opened and valve 5 is adjusted so as to connect the manifold with the connection linking valve 5 with a bulk starter vessel V. As the steam entering the container through the valve 3 is under pressure, the liquid contents of the container will be forced to travel up the pressure tube 13, through valves 4 and 5 and into the bulk starter vessel. The position and length of the pressure tube 13 inside the container is such that some of the contents of the container will, however, always remain behind in the bottom of the container so that they can serve as inoculum for the next mother culture to be propagated in the container.
- In this way, an aseptic transfer of most of the mother culture is effected from the container M to the bulk starter vessel V. The latter is then isolated from the container by closing the connection between the valve 5 and the vessel. The container is likewise isolated from the rest of the system by closing its valves 3, 4.
- The passages from the valves 3 and 4 to the valve 5, to the air filter isolating valve 6 and, if desired, the autoclave and its connecting passages are then rinsed with water and flushed with suitable washing and cleansing liquids in order to remove the residues of the liquid medium or mother culture. This rinsing is effected by connecting the autoclave and the various valves and passages to a supply of rinsing liquid via the valve 2 and flushing through, preferably under pressure.
- After the transfer of most of the mother culture from the container M to the vessel V and the flushing of the connections, the passages connecting the autoclave to the container valves 3, 4 are purged of air and sterilized by generating steam in the autoclave and allowing this steam to circulate through the various connections so as to force air out to atmosphere through the air bleed in the valve 5, thereafter closing the air bleed and maintaining at least 15 p.s.i. steam gauge pressure for at least 15 minutes whilst allowing the steam to pass through valve 5 on its way to the steam bleed outlet in relief valve 18.
- After this sterilization procedure, the valve 1 is adjusted so that the steam outlet passage 19 is connected to the bleed in valve 1 thus isolating the autoclave from the valve and passage system. The autoclave is then opened and filled with sufficient liquid medium for the growth of a new mother culture. The autoclave is then reclosed and the medium sterilized in the manner described above. The sterilized liquid medium is then cooled and transferred to the container M through the previously steam-sterilized connecting passages and valves. Some of the contents of the previous mother culture left over in the container are thus mixed with the fresh batch of sterilized liquid medium and a new mother culture can now be grown after isolating the container, aerating it if necessary as described above and maintaining it at temperatures suitable for the growth of the selected microbial culture for the time required for the development of a new microbial population. During this incubation period, the container M can be supplied, if required, with sterile air or gas by means of its connection with the air or gas filter.
- On completion of the incubation, the air

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- or gas supply to the container M is closed off and the new mother culture can be transferred to the appropriate bulk starter vessel V in the manner described above.
- 5 Alternatively, after closing the valves 3, 4, the container and its associated manifold can be detached from the rest of the system and the new mother culture stored in a deep-frozen condition for use at a later date.
- 10 Another mother culture may then be brought out of storage, its container attached to the system, the culture thawed if necessary, and then transferred aseptically to the appropriate starter vessel in the manner described above.
- 15 It is evident from the above description that when more than one strain or type of micro-organism is used, each of the strains or types has its own container M. The scheme thus permits of a multi-strain or multi-culture rotation.
- 20 Valves 1, 2 and 5 and their interconnecting passages are preferably combined in a single valve block assembly. The valves may be operated manually or in some other way such as pneumatically. If they are operated pneumatically, the sequence of operations may be automatically controlled by means of an electrically operated programme controller, a system of electrical interlocks being provided to ensure that the timing system operates correctly.
- 25 The process as described in detail above will be seen to comprise the following features:—
- 30 (a) Sterilization of the apparatus and medium for the growth of the micro-organisms of the mother culture;
- 35 (b) Aseptic transfer of sterilized medium to a sterilized mother culture container connected to the sterilizing vessel;
- 40 (c) Aseptic initial inoculation of the sterilized medium in the mother culture container;
- 45 (d) Aseptic incubation of the inoculated mother culture;
- 50 (e) Aseptic transfer of the incubated mother culture to the bulk starter vessel;
- 55 (f) Retention of part of the mother culture in its container to serve as an inoculum for the next mother culture;
- 60 (g) Supply of sterile medium under aseptic conditions to the mother culture container which has retained an inoculum from the previous mother culture;
- 65 (h) Aseptic incubation of the new mother culture;
- (i) Preservation by deep-freezing of the mother culture in its container until it is required in the scheme of rotation for the inoculation of a bulk starter;
- (j) Aseptic transfer of the culture of the bulk starter after conversion from the frozen to the liquid state;
- (k) Aseptic preparation and preservation
- of a new mother culture in the manner described under (f), (g), (h) and (i).
- More generally, the invention will be seen to embody the steps of aseptically transferring sterilized medium to a sterilized mother culture container, inoculating the medium in the container under aseptic conditions, incubating the inoculated mother culture under aseptic conditions, transferring portion of the incubated mother culture to another vessel under aseptic conditions, retaining portion of the incubated mother culture in the container to serve as an inoculum for a fresh culture, supplying fresh sterilized medium under aseptic conditions to the container, and incubating the fresh mother culture thus formed under aseptic conditions.
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- It will be apparent that the above described specific embodiment of the apparatus of the present invention substantially eliminates the risk of contamination during inoculation at the mother culture stage and at the transfer of the mother culture to the bulk starter vessel. The specifically described method of carrying out the process of the invention also has the advantage of prolonging the viability of the micro-organism concerned by permitting a culture of the micro-organism to be stored in a deep-frozen state. A further advance made possible by the said embodiment of the apparatus of the invention resides in the automatic preparation of mother cultures in sterile detachable vessels. Accordingly, it will be clear that the invention will enable considerable advances to be made in many industrial fermentation processes and particularly in the dairy products industry concerned in the manufacture of cheese and like products.
- WHAT WE CLAIM IS:—**
1. A process for the aseptic propagation of microbial cultures comprising the steps of aseptically transferring sterilized medium to a sterilized container, inoculating the medium in the container under aseptic conditions, incubating the inoculated culture under aseptic conditions, transferring portion of the incubated culture to another vessel under aseptic conditions, retaining portion of the incubated culture in the container to serve as an inoculum for a fresh culture, supplying fresh sterilized medium under aseptic conditions to the container, and incubating under aseptic conditions the fresh culture thus formed.
 2. A process for the aseptic propagation of microbial cultures comprising the steps of aseptically transferring sterilized medium to a sterilized container, inoculating the medium in the container under aseptic conditions, incubating the inoculated culture under aseptic conditions, passing a sterile vapour under pressure into said container to cause por-

- tion only of the incubated culture to be transferred to another vessel, retaining portion of the incubated culture in the container to serve as an inoculum for a fresh culture, supplying fresh sterilized medium under aseptic conditions to the container, and incubating under aseptic conditions the fresh culture thus formed. 40
3. A process as claimed in claim 1 or 45
claim 2, and further comprising the step of sealing the container after incubation of the fresh culture therein and freezing the incubated culture in the sealed container to preserve the culture until required for use. 50
4. A process as claimed in claim 3, and further comprising the step of passing a sterile vapour under pressure into said container to cause portion only of the thawed-out culture to be transferred to another vessel. 55
5. Apparatus for the aseptic propagation of microbial cultures comprising sterilizing means, a container suitable for the growth of microbial cultures, connections between the container and a vessel into which a culture grown in the container can be passed, connections between the sterilizing means and the container, and valve means in said connections, the arrangement being such that both aseptic propagation of microbial cultures within the container and the aseptic transfer of microbial cultures from the container to the vessel can be effected, the connections between the container and the vessel being arranged so as to ensure that a portion of the contents of the container remains in the container when a culture is 60
6. Apparatus as claimed in claim 5, in which the connections between the container and the vessel comprise a tube the lower end of which is spaced from the bottom of the container. 65
7. Apparatus as claimed in claim 6, in which the connections from the container to the vessel and from the container to the sterilizing means enable the container to be disconnected from the system and re-connected thereto when desired, the said valve means enabling both said connections to be put into communication with the sterilizing means and thereby sterilized after re-connection of the container.
8. Apparatus as claimed in any one of claims 5 to 7, and including a gas sterilizing filter for the admission of sterile gas to the container. 55
9. A process for the aseptic propagation of microbial cultures substantially as hereinbefore described with reference to the accompanying drawings. 60
10. Apparatus for the aseptic propagation of microbial cultures constructed and arranged substantially as hereinbefore described and as shown in the accompanying drawings. 65

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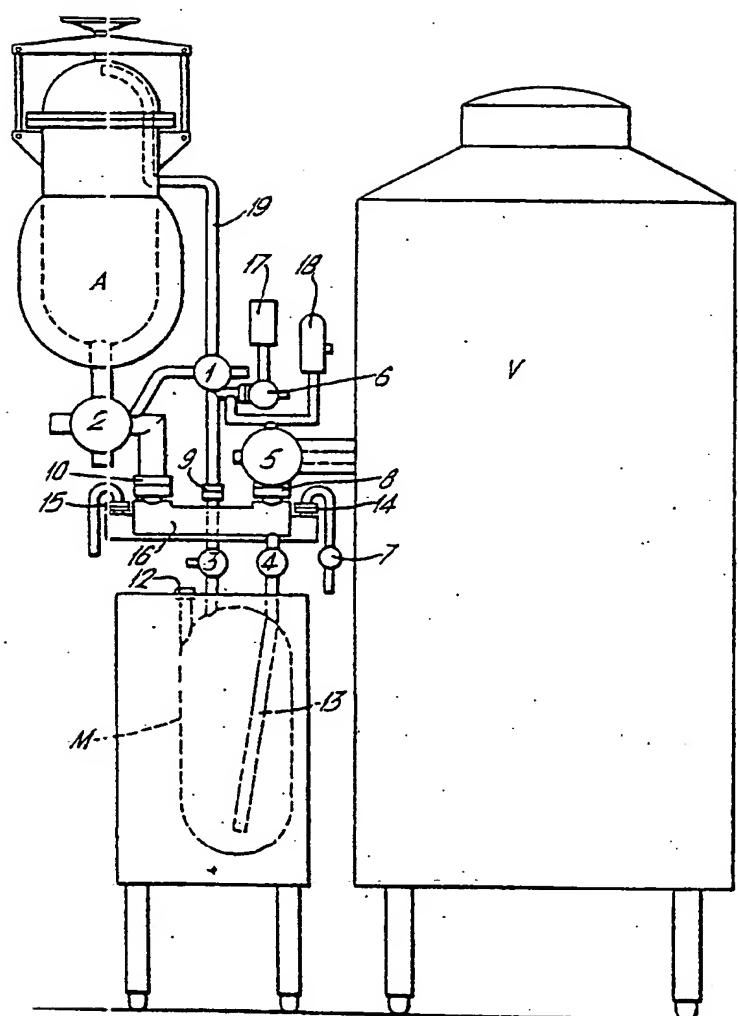
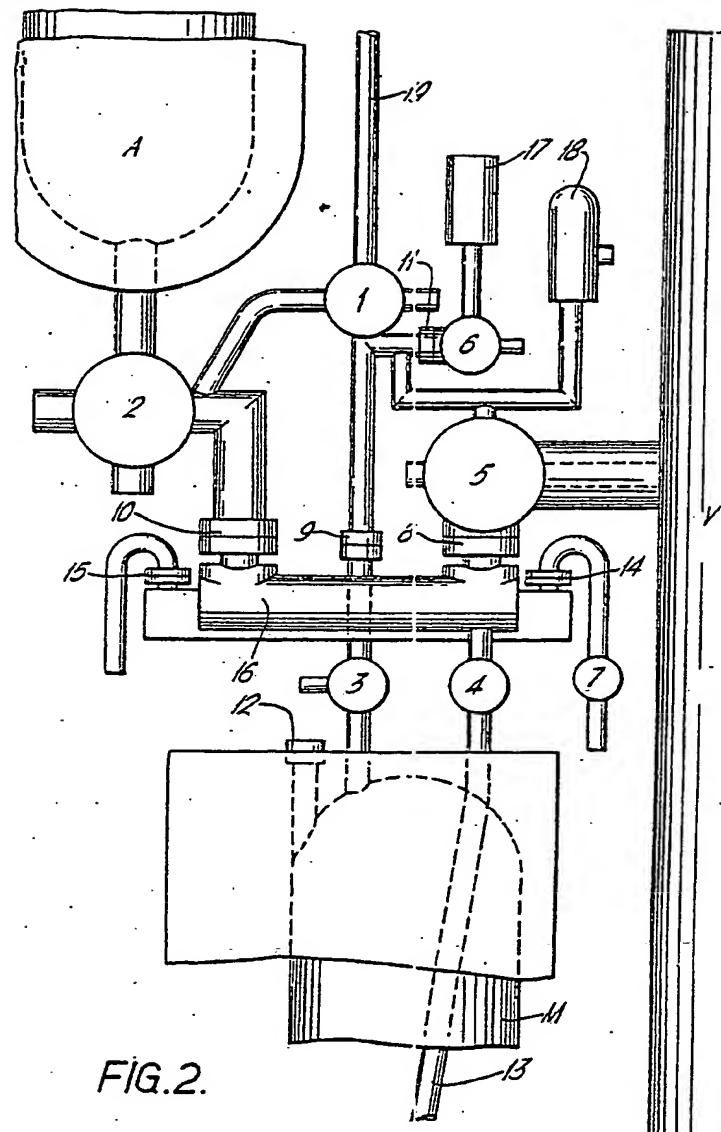


FIG. 1.

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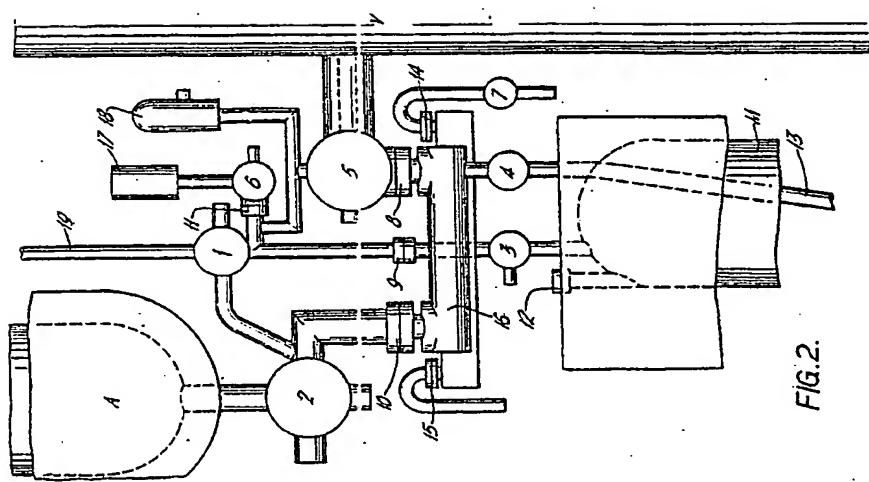


FIG. 2.

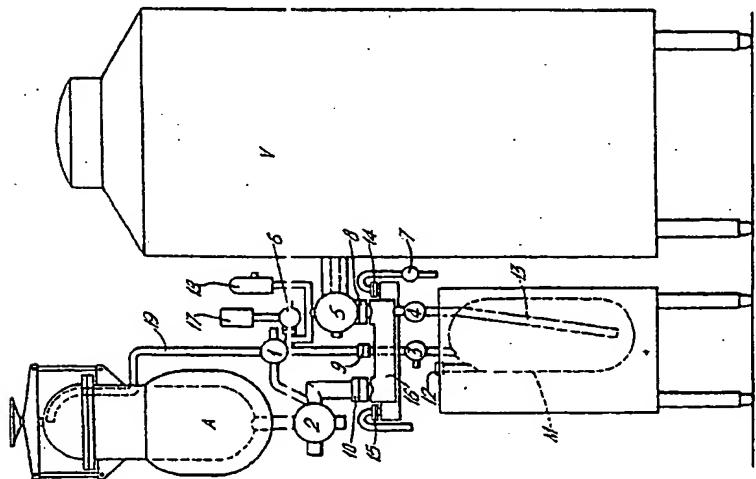


FIG. 1.

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